The regulation of self-reactive B cells

Richard J Cornall, Christopher C Goodnow and Jason G Cyster

Howard Hughes Medical Institute and Stanford University School of Medicine, Stanford and University of California, San Francisco, USA

Self-reactive B cells are eliminated in a series of checkpoints that are triggered by antigen binding. Recent reports have shown that in addition to the processes of elimination at the immature B-cell stage, B-cell anergy and regulation of T-cell help, self-reactive cells are also controlled by follicular competition, Fas-mediated elimination by T cells and censoring in the germinal centres. Each checkpoint operates at a threshold that reflects the need to maintain immune diversity at the same time as suppressing autoimmune disease. Analysis of the *motheaten* mutation has given a direct demonstration of how such thresholds can be modulated by genetic effects.

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Introduction

The immune system has the ability to produce a diverse repertoire of antigen-specific lymphocytes capable of responding to an unlimited array of pathogens, but at the same time remain unresponsive to self-molecules. This review summarizes work during the past year that contributes to our current understanding of the checkpoints at which self-reactive B cells are either eliminated or inactivated (see Fig. 1), and then considers the ways in which the threshold for these outcomes can be influenced by genetic and environmental events.

Regulation of self-reactive cells in the preimmune repertoire

Checkpoint 1: elimination of immature B cells

Experiments with transgenic mice have shown that when a multivalent membrane-bound antigen binds to immature B cells in the bone marrow, the cells are eliminated [1]. The elimination is preceded by both profound downregulation of surface (s)IgM expression and maturation arrest [2]. During maturation arrest, the self-reactive cells may escape deletion by converting to non-autoreactive specificities, either by editing light chain (Lc) alleles or, more rarely, by deleting the transgenes. These events, and those of other checkpoints, have recently been reviewed in detail [3•].

During that past year, Weigert and colleagues [4••] described the deletion of immature B cells expressing an anti-dsDNA heavy chain (Hc) transgene exposed to autoantigen in the bone marrow. Escape from censoring was prevented by introducing mutations into the Hc transgene, which created dsDNA-reactive antibodies when paired with almost any Lc, and by breeding the transgene onto mice in which the endogenous Hc was disrupted by a targeted mutation of the JH region.

The efficiency with which membrane-bound antigen can cause the deletion of self-reactive cells ensures that there is robust tolerance to a range of abundant membrane-bound antigens, particularly those likely to be encountered on haemopoietic cells and blood-exposed organs. Transgenic mice that express an anti-hen egg lysozyme (HEL) Hc transgene, but no HEL antigen, possess small subsets of HEL-binding B cells with a range of binding affinities varying over four orders of magnitude. Exposure to membrane-bound HEL (mHEL) during development deletes even the lowest affinity cells in these mice [5•]. In complementary experiments, Lang and Nemazee [6•] found that B cells bearing anti-K^k antibody are effectively deleted even when exposed to a range of MHC class I molecules for which the antibody has low binding affinities.

One criticism of transgenic experiments has always been that the B cells may be abnormal as a result of their accelerated development. Reassuringly, by following the relative representation of an endogenous variable (V) region gene, VH81X, which is known to be highly self-reactive in many Lc combinations, Klinman and colleagues [7•] have extended earlier observations to show relative under-representation of VH81X-bearing cells among the sIg^+ cells in the spleen, compared to sIg^- pre-B cells in the bone marrow, and an intermediate representation amongst sIg^+ immature B cells in the bone marrow.

Although many self-reactive B cells do encounter multivalent antigen in the bone marrow, it is yet to be confirmed that this is the site of their deletion. Hartley and Goodnow [8•] were unable to obtain histological

Abbreviations

APC—antigen-presenting cells; **DEL**—duck egg lysozyme; **FDC**—follicular dendritic cell; **Hc**—heavy chain; **HEL**—hen egg lysozyme; **IL**—interleukin; **Lc**—light chain; **sIgM**—surface IgM; **V**—variable.

evidence for the apoptosis of self-reactive B cells in the bone marrow because of the high background of apoptotic cells from various haemopoietic-cell lineages. The best indirect evidence for elimination at this site probably remains the marked accumulation in the bone marrow of cells under developmental arrest, which have been saved from apoptosis by their expression of a *Bcl-2* transgene [2]. Several recent studies have attempted to address this question by showing the loss of B-cell populations in the bone marrow which have been separated on the basis of sIgM expression [7•,9•]. It is an important practical point, however, that antigen binding itself causes downregulation of sIgM, so that it may not be possible to use the level of sIgM expression to separate immature from more mature B cell sub-populations with any accuracy. In a polyclonal mixture, the subset of more mature B cells may carry greater amounts of sIgM, not as a result of maturation, but because avidly self-reactive B cells are depleted from this pool by sIgM modulation and developmental arrest. For example, in the anti-HEL transgenic model, the binding of high affinity sIgM to mHEL results in a 50-fold decrease in the intensity of staining for sIgM, in addition to halting maturation at the $IgD^-CD21^-CD23^-$ stage [2,8•].

These considerations aside, it is nevertheless likely that some self-reactive B cells only meet their autoantigen and become deleted after leaving the bone marrow [9•]. New evidence that membrane-bound self-antigen can cause deletion outside of the bone marrow comes from a transgenic model in which B cell antigen receptors bind to the T cell allelic autoantigen CD8.2 [10••]. Although only a few mature follicular B cells are present in the spleens of these animals, immature B cells in the bone marrow and immature recent emigrants to the spleen are present in normal numbers. It is likely that the failure to detect any effect of membrane antigen in the bone marrow is a result of the low chance of the self-reactive B cells interacting with CD8 T cells.

The molecular basis for the elimination of immature B cells remains unclear, although it is probably independent of Fas [11•]. Differences in antigen receptor signaling between immature and mature B cells may increase the tendency for immature cells to undergo apoptosis, rather than mitosis, following receptor engagement, at least *in vitro* [12•].

Autoimmune disease can develop when B cells fail to encounter antigen at the immature stage. This is illustrated by the anti-erythrocyte transgenic model of Honjo and colleagues [13] that predisposes towards the formation of peritoneal B-1 cells, which are hidden from autoantigen and are neither eliminated nor modulated at their sIgM receptors unless self-erythrocytes are introduced artificially into the peritoneal cavity. Under normal circumstances, half of these transgenic mice develop spontaneous haemolytic anaemia which is strongly associated with the activation of B-1 cells. Murakami *et al.* [14•] have now shown that the oral administration of lipopolysaccharide activates the peritoneal B-1 cells and promotes haemolytic anaemia in all cases. In a similar manner, enteric T cell independent antigens or polyclonal activators from bacteria might circumvent T-cell tolerance and cause autoimmune disease. Such a mechanism could explain haemolytic anaemia in a child lacking MHC class II expression [15].

Checkpoint 2: follicular competition

A large number of B cells arrive daily at the spleen and when it is saturated, most disappear within one to three days, after reaching the T-cell zones, but without entering B-cell rich primary follicles [16]. This result, together with the skewing of antibody V region usage that occurs between immature bone marrow B cells or neonatal B cells and mature B cells [17], suggests that only B cells with particular specificities can survive at this site.

Because it is impossible to examine the relative fates of different B-cell clones in a uniform transgenic B-cell population, Cyster *et al.* [18••] followed anti-HEL transgenic B cells mixed with non-transgenic B cells of polyclonal specificities. In the absence of autoantigen, the anti-HEL B cells followed the normal route into the primary follicles from the T-cell zones as effectively as competing B cells of other specificities, and were able to recirculate in the mouse and survive like non-transgenic cells. Under the same competitive conditions, however, the HEL-binding B cells continuously exposed to anergizing concentrations of soluble HEL

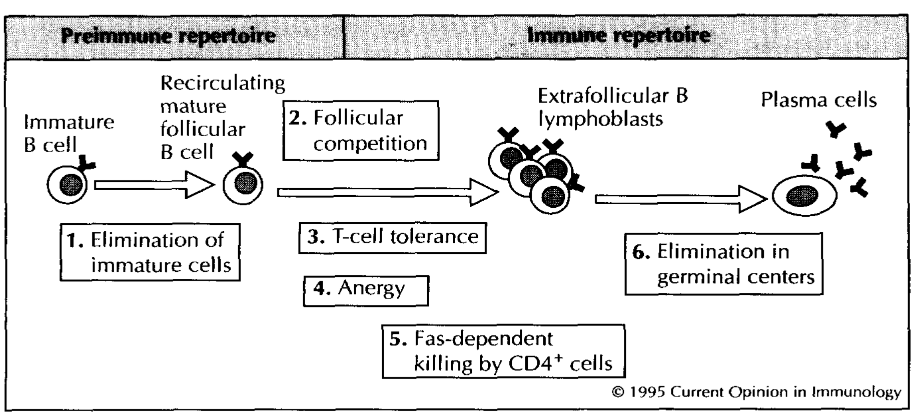

Fig. 1. Self-tolerance checkpoints in B-cell development. Maturing B cells encounter a series of checkpoints in the preimmune and immune repertoire at which self-reactive cells are either eliminated or inactivated. Only cells that can successfully pass through all of these checkpoints will go on to become antigen-secreting plasma cells.

(sHEL) autoantigen were excluded from the follicles and died within less than three days. Exclusion from the follicles was a result of competitive phenomenon rather than a result of binding of autoantigen alone, because autoantigen-bound transgenic B cells could recirculate and survive normally in the absence of a sufficient number of unbound competitors. Furthermore, failure to enter the follicles was not a result of cell death because sHEL-bound self-reactive B cells expressing a *Bcl-2* transgene had enhanced survival in the T-cell zones but remained excluded from the follicles [18••].

Competition for follicular niches and the survival factors available there, provides an explanation for how self-reactive B cells binding soluble or low-avidity autoantigens can be censored in the secondary lymphoid organs, and why, in a situation of inadequate competition, immunodeficiency can result in autoimmunity. An example of this was provided in a recent report by Young and Kearney [19•], who found that there was a greatly elevated frequency of strongly self-reactive cells in the small peripheral B-cell population of severe combined immunodeficiency (SCID) mice. Differential competition for follicular niches, rather than autoantigen binding *per se*, is also likely to explain the 3.5 day lifespan of anergic cells found by Fulcher and Basten [20•], who compared the survival of anti-HEL transgenic B cells in mice expressing autoantigen with that of competing non-HEL-binding B cells in the same animals. Although 40% of both antigen-bound and unbound cells in six week old mice survived for two weeks, animals of 12 weeks and older showed a progressively more rapid turnover of the B cells bound to autoantigen. This effect is likely to a result of follicular competition between the antigen-bound transgenic B cells and cells expressing endogenous sIg receptors, which accumulate in older mice. As the survival of anergic B cells even in young mice with few competing B cells is still not as long, on average, as that of most non-transgenic cells, a direct effect of autoantigen on the lifespan of anergic B cells may also exist.

The regulation of self-reactive cells in the immune repertoire

Checkpoint 3: T-cell help

The fact that excluded anergic B cells (and primed cells from germinal centres — see later) die in the T-cell zone raises the question of whether these cells can be rescued by T-cell help. Evidence in support of this possibility comes from *in vitro* studies in which B cells that have been removed from the follicular stroma and exposed to anti-Ig antibodies are rescued from apoptosis by concurrent stimulation with mitogens or CD40 ligand [21•,22,23]. The extent to which apoptosis is triggered by withdrawal from stromal growth factors, or by sIg signaling itself, is unknown, although like the elimination of less mature cells in the bone marrow, death by follicular exclusion *in vivo* is delayed by the expression of *Bcl-2* and is independent of normal expression of Fas ([21•]; JG Cyster, JC Rathmell, CC Goodnow, unpublished data). The capacity for T-cell help to rescue a short-lived excluded B cell depends upon two controls: first, the state of T cell tolerance to the relevant antigens; and second, the ability of the B cell to present antigenic peptide and costimuli and collaborate effectively. As we shall see, the ability of a B cell to present costimuli and collaborate with helper T cells depends upon the duration and timing of the B cell's exposure to antigen.

The capacity for T-cell help to rescue B cells and promote their proliferation after acute antigen receptor engagement has been demonstrated in the mouse polyclonal repertoire by Finkelman *et al.* [24] who used goat anti-IgD antibodies to simultaneously cross-link B cell antigen receptors and to recruit help from goat IgG specific T cells. By contrast, treatment with rat monoclonal anti-IgD antibodies, after deleting helper T cells and blocking further B-cell development with anti-IL-7, leads to deletion of many peripheral B cells [25•]. The B cells die slowly over a period of five days to leave a population of cells that bind with less avidity. The deletion of a sub-population of murine B cells expressing transgenic IgM rheumatoid factor by deaggregated human IgG seems likely to be caused by a similar mechanism [26••]. Whether chronic sIg receptor cross-linking in the absence of T-cell help triggers deletion directly or by excluding the subset of triggered B cells from access to the follicular stroma remains to be determined.

Checkpoint 4: anergy

Activated B cells can be excellent antigen-presenting cells (APCs) [27•–29•]. B cells which have been chronically exposed to antigen during their development, however, differ from naive cells in their APC function [27•,30••] and appear poorly able to generate or receive T-cell help. This situation arises when autoreactive cells are recurrently exposed to an autoantigen, such as soluble circulating lysozyme, which is of insufficient avidity or affinity to trigger deletion in the bone marrow, but sufficient avidity to affect the cell's ability to compete for follicular entry and its capacity to be activated by antigen. Such cells are termed anergic, and, in the absence of follicular competition, they are long-lived. The notion that anergy is a normal part of the spectrum of response to binding of autoantigen by immature B cells is buttressed by data from transgenic mice bearing anti-DNA antibodies with particular affinities and/or fine specificities [31,32].

The functional inactivation of anergic B cells has been extensively investigated in the lysozyme model, and comprises both a five to 20-fold downmodulation of the expression of sIgM [33•] and a proximal desensitization in sIg signaling with greatly diminished tyrosine kinase activation and calcium flux in response to binding of HEL [30••]. Despite normal processing of antigen and presentation by the MHC class II complex, and an ability to respond normally to T-cell mediators IL-4 and anti-CD40 *in vitro*, anergic cells fail to

generate T-cell help, at least in part because of defective CD28-dependent costimulation [27•,29•,30••,34•,35•]. Inadequate expression of CD86 (B7.2/B70) in response to antigen binding is caused by the block in receptor signaling [30••].

Acute exposure to multivalent membrane-bound lysozyme can partially restore signaling in anergic B cells and induce proliferation and differentiation in the presence of T-helper cells *in vivo* [30••]. This suggests that anergic B cells might be transiently rescued into antibody production if they bind more avidly to multivalent pathogenic antigens than they bind to non-pathogenic autoantigens, and that this trade-off would be a rationale for allowing short-lived, but potentially self-reactive, cells to emigrate from the bone marrow into the T-cell zones of secondary lymphoid tissues.

Checkpoint 5: Fas-mediated elimination of anergic cells

It is conceivable that, through complexing or cross-reaction between self and foreign antigens, anergic self-reactive B cells might encounter competent T-cell help and be effectively costimulated through CD40, despite the absence of adequate sIg antigen-receptor mediated signaling. Rathmell *et al.* [36••] have investigated this possibility using *in vivo* transfer to mix anergic anti-HEL B cells with non-tolerant HEL-specific TCR transgenic $CD4^+$ T cells and have found that the anergic B cells are deleted when they present autoantigen. Unlike the premature death that occurs following follicular exclusion in the absence of T-cell help, T-cell dependent peripheral deletion is mediated by Fas. B cells carrying the Fas mutation, *lpr*, are not deleted and T cells carrying the Fas-ligand mutation, *gld*, are unable to trigger the deletion process. By contrast, non-tolerant B cells are induced to proliferate and secrete antibody in response to antigen and T-helper cell exposure despite the induction of Fas expression ([36••]; JC Rathmell, CC Goodnow, unpublished data). The polarized fates of anergic and immune-competent B cells are likely to be determined by their differences in antigen-mediated cell signaling pathways. Acute antigen-receptor engagement with anti-IgM can signal protection against Fas-dependent apoptosis mediated by T-helper cells type 1 [37••], an effect which might be blocked in anergic B cells.

This mechanism provides clues to the pathogenesis of autoimmune disease in MRL/*lpr* and *gld* mice and also in the recently described human cases of Fas deficiency [38••,39••]. It may also explain reports of suppressor function mediated by class II restricted lymphocytes [40,41].

Checkpoint 6: elimination in germinal centres

The majority of B cells activated in the T-cell zones migrate to local sites of antibody production (the red pulp in the spleen and the medullary cords in the lymph nodes) where they differentiate into short-lived plasma cells. At the same time, a minority of the cells enter the follicles where they form germinal centres and undergo class switching and somatic hypermutation, possibly to autoreactive specificities.

Previous studies have suggested that somatically mutated B cells in germinal centres undergo apoptosis unless they receive survival signals at two control points: first, an antigen-receptor mediated signal from a high-affinity interaction with immune complexes on the surface of specialized follicular dendritic cells (FDCs); and second, CD40 costimulation, which is once again received by effective interaction with non-tolerant T cells (reviewed in [42•]).

Direct visualization of B-cell censoring in the germinal centres *in vivo* has been demonstrated in two recent reports [43••,44••]. Shokat and Goodnow [44••] tracked the fate of transgenic anti-HEL B cells seeded into the developing germinal centres of non-transgenic mice immunized with duck egg lysozyme (DEL). After five days, the seeded cells filled the germinal centres. The mice were then exposed to soluble HEL, which binds the transgenic B cells with 1000-fold greater affinity than DEL but does not cross-react with DEL at the level of T-cell help. The effects of soluble antigen in the absence of T-cell help were that a wave of apoptosis occurred within four hours in the germinal centre, followed by a relocation of other B cells from apical follicular and marginal zones to outer T-cell zones and a second wave of apoptosis at 12 hours within the T-cell zones. Strikingly similar observations were made by Pulendran *et al.* [43••] who immunized unmanipulated mice with the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to human serum albumin (HSA) in adjuvant, and then, at the height of the germinal centre response, observed the effects of the same NP antigen but in high dose soluble form and linked to immunogenic or self-protein carriers. The effect of soluble antigen in the absence or presence of T-cell help was a wave of apoptosis within the germinal centre peaking at four hours.

These results are consistent with the model of two germinal centre selection points: at the first, soluble antigen actively or passively antagonizes transmission of survival signals from interactions between centroblasts and antigen-bound FDCs; at a later stage, those centrocytes that have bound soluble antigen, relocate to the T-cell zones in a manner reminiscent of follicular exclusion, where their potential to escape apoptosis and propagate B-cell memory is again determined by T-cell help. Exposure to soluble autoantigen would thus be expected to cause the deletion of self-reactive cells that arise by hypermutation. Absence of censoring at this stage accompanies autoimmunity, as illustrated by patients who develop pathogenic hypermutated rheumatoid factor antibodies despite the continuous presence of soluble autologous immunoglobulin [45•].

Thresholds of self-tolerance

Whether or not any particular self-reactive B cell is censored at each checkpoint will be determined by a

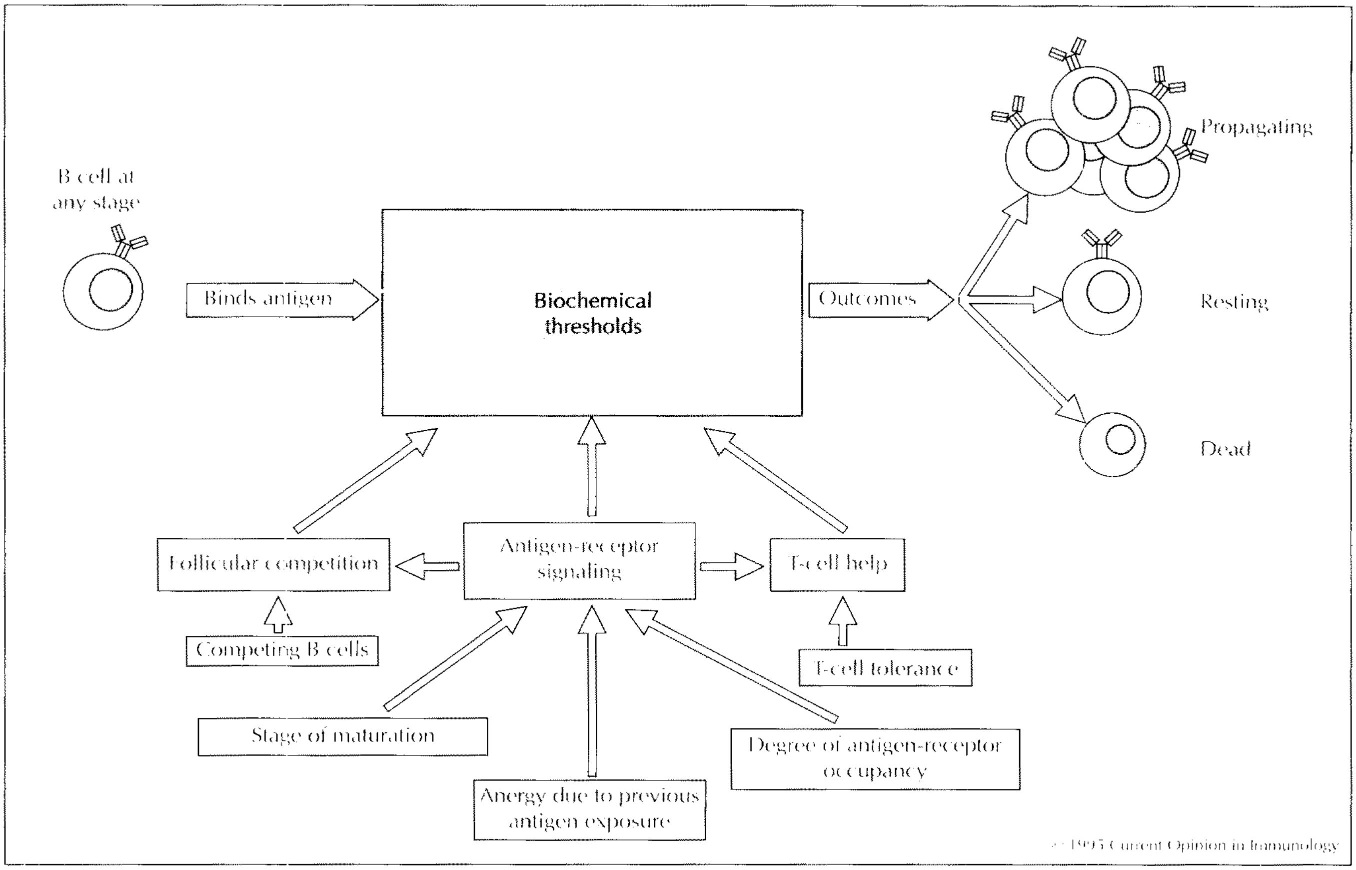

Fig. 2. The fate of antigen-bound B cells is determined by several variables. Antigen-receptor engagement may result in B-cell proliferation and maturation, maintenance in a resting state, or B-cell death. The choice between these outcomes is determined by biochemical thresholds that receive inputs from the variables shown by the boxes and arrows. Much work is needed to define the biochemical basis of these choices and how they vary according to genetics and environment.

biochemical triggering threshold that has presumably arisen in order to maintain immune diversity at the same time as suppressing autoimmune disease. The biochemical strength and quality of antigen-receptor signaling determines whether or not the triggering threshold is achieved, thus integrating antigen valency, antigen concentration, and receptor affinity, as well as the history of the individual B cell and the effects of other cells that comprise its environment.

Direct evidence for the importance of signal strength comes from analysis of *motheaten* viable (*me^v*) mice, which are deficient in the cytosolic tyrosine phosphatase PTP1C (also called SHP, HCP and SHPTP-1) and exhibit severe immunodeficiency and autoantibody production. Cyster and Goodnow [46••] found that antigen triggers a greater and more rapid elevation of intracellular calcium in B cells deficient in PTP1C, indicating that the phosphatase negatively regulates an early step in immunoglobulin signaling. In the absence of HEL autoantigen, PTP1C-deficient B cells [46••] nevertheless assume an anergic phenotype, which is consistent with chronically increased spontaneous signaling in the immunoglobulin pathway; and, in the presence of soluble HEL autoantigen, they undergo exaggerated sIgM modulation, maturational arrest, and elimination in the bone marrow in a manner similar to normal B cells exposed to the more potent membrane-bound autoantigen

Biochemical experiments show that PTP1C is recruited to the cytoplasmic domain of CD22, a co-receptor molecule which becomes tyrosine phosphorylated by activated antigen-receptors [47••]. Local activation of the phosphatase's activity may therefore introduce a negative feedback loop by opposing the activity of receptor-associated tyrosine kinases. PTP1C has also been found to physically associate with the resting antigen-receptor complex in a lymphoma cell line, from which it may dissociate during the initial stage of acute receptor activation [48•]; it is also likely to play a role in immune complex-mediated inhibition of antigen-receptor signaling, as it rapidly associates with FcγRIIB1 which has been coligated to the antigen-receptor complex [49••].

We have seen how several variables combine with the strength of immunoglobulin signaling to determine the fate of B cells bound to antigen (see Fig. 2). It is reasonable to speculate that other polymorphisms affecting the strength and quality of immunoglobulin signaling will be important contributors to genetic susceptibility or resistance to autoimmune disease. It is increasingly evident that the predisposition to

common autoimmune diseases in humans occurs when different combinations of susceptibility alleles combine to reach an overall susceptible threshold. This point is illustrated by the recently described human autoimmune lymphoproliferative disorder which is associated with Fas mutations. This disease is of early onset in childhood and therefore likely to be strongly genetic in origin. However, the parents of affected individuals who also carry the mutation are unaffected, suggesting that, as in MRL/*lpr* mice, other background susceptibility genes are required for the Fas mutations to result in disease [38••,39••]. Multiple additive susceptibility alleles have also been found in murine models of lupus [50•–52•]. It is easy to conceive how such variants could arise within the self-tolerance pathways described in this review and, under most circumstances, have neutral and occasionally even advantageous effects by favouring rapid responses to foreign pathogens. By defining the biochemical basis of each checkpoint in detail, it will be possible to test directly for the nature and effects of these variants.

Conclusions

It is now established that activation of the B cell antigen receptor causes survival, proliferation, inactivation, elimination, or no effect, depending upon circumstances, which include the maturity and location of the B cell, the strength of the signal, and the effects of other cells including T cells and other B lymphocytes. Each of these outcomes has its own threshold, which may be affected by genetic and environmental influences. Autoimmune disease may arise by a multistage and multifactorial process when changes in the thresholds of several checkpoints allow clones of self-reactive cells to escape negative selection and produce autoantibodies.

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RJ Cornall and CC Goodnow, Howard Hughes Medical Institute and Department of Microbiology and Immunology, Beckman Center, Stanford University School of Medicine, Stanford, California 94305, USA.

JG Cyster, Department of Microbiology and Immunology, University of California, San Francisco, California 94143-0414, USA.